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BICHEMICAL CHANGES AND OXIDATIVE DAMAGE IN *AZOLLA PINNATA* L. UNDER CHROMIUM PHTOTOXICITY STRESS

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SUMMARY

We studied the effect of both Cr^{3+} and $Cr_2O_7^{2-}$ on lipid peroxidation and different antioxidative enzymes, in fronds of *Azolla pinnata* L. in order to know the possible involvement of this metal in the induction of oxidative stress leading to biochemical and oxidative aberrations. Chromium treatment for 2, 4 or 6d resulted in decreased biomass of fronds. A significant increase in activities of superoxide dismutase (SOD), peroxidase (POX) and glutathione reductase(GR) as well as non-enzymic antioxidants, indicated accumulation of hydrogen peroxide and oxidative stress. The increase in superoxide dismutase activity with decline in catalase activity in *A. pinnata* indicated a sensitive biomarker in monitoring aquatic contaminants like chromium.

Key words: **Antioxidants, *Azolla pinnata*, chromium, lipid peroxidation, oxidative stress.**

INTRODUCTION

Chromium is a non-essential element for plants. In nature chromium exists as Cr(III) and Cr(VI), of which Cr(VI) is highly toxic to the terrestrial and aquatic organisms. Chromium is considered as a major pollutant of air, soil and water due to its widespread use in leather tanning, pigments, electroplating and various alloys. Chromium phytotoxicity can result in degradation of pigments, antioxidant enzymes and induce oxidative stress in plants (Upadhyay and Panda 2003, Sanita di Toppi *et al.* 2004). As an important water contaminant, chromium induces severe cellular damage, changes in nutrient content and enzyme activities of various crops and aquatic plants (Shi *et al.* 2003, Arora *et al.* 2006). It has been reported in diverse group of plants that chromium initiates the activation and regulation of enzymes that are involved in the plant defense systems (Upadhyay and Panda 2004). Both forms of Cr differ in terms of mobility, bioavailability and toxicity (Panda and Choudhury 2005). Chromium is widely distributed in nature and acts as catalyst in the formation of reactive oxygen species (ROS) which have the capacity to initiate lipid peroxidation and degrade proteins, lipids and nucleic acids (Scandalios 1993, Hernandez *et al.* 1994, Weckx and Clijisters 1997). These ROS produced excessively under stressful conditions are removed by complex non–enzymatic (ascorbate, glutathione, a-tocopherol) and enzymatic (catalase, guaiacol peroxidase, superoxide dismutase, glutathione reductase etc.) antioxidant systems (Bartosz 1997, Prochazkova *et al.* 2001, Kuo and Kao 2003, Gratao *et al.* 2005). The activities of all of these antioxidant enzymes are induced with response to adverse biotic and abiotic stresses (Foyer *et al.* 1994). Changes in functioning of the antioxidative system are also known upon heavy metal stress (Stroinski 1999). The antioxidative system consisting of several enzymes and antioxidants fulfill a protective role and stabilize the amount of ROS in plant cells (Salin 1987). *Azolla pinnata* L. is a fast growing aquatic floating microphyte

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and one of the important components of the aquatic ecosystem (Arber 1963, Culley *et al.* 1981) which are affected by anthropogenic activities. Though much is known about the biology of *Azolla*, very little information is available about its sensitivity to chromium. The present investigation was undertaken to test the hypothesis that chromium toxicity induces oxidative damage in aquatic microphytes and results in the possible involvement and induction of antioxidant system.

MATERIALS AND METHODS

Plant material and growth condition: Azolla pinnata L. plants, collected from uncontaminated pond (20°04' N, 90°40' E) were washed with double distilled water several times and soaked dry without damaging the tissues, and grown under laboratory conditions at 25 °C. Five plants were transferred to sterile petri plates with different concentrations (0,0.01,0.1,1,10mM) of both Cr(III)[Chromium oxide] and Cr (VI)[Potassium dichromate] solution with three replicates each. The petri plates, with 10 ml solution each were incubated under white fluorescent tube lights (52 $\mu molm^{-2}s^{-1}$) for 2, 4 and 6 d at 25±2 °C. After the treatments the fronds were sampled for various biochemical and enzymatic investigations.

Determination of Lipid per oxidation and H_2O_2 content: The lipid peroxidation content was determined as thiobarbituric acid reactive substances, as described by Heath and Packer (1968). Frond tissues (0.2g) were extracted in 2 ml of 0.25% thiobarbituric acid (TBA) made in 10% trichloroacetic acid. The extract was heated at 95 °C for 30 min and then cooled on ice. After centrifugation at 10 000 g for 10 min, the absorbance of the supernatant was measured at 532 nm and correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm using UV-visible spectrophotometer (Systronics,Gujarat,India). The level of lipid peroxidation was expressed as mmol of malondialdehyde g^{-1} fw using an extinction coefficient of 155 mM cm^{-1}.

The H_2O_2 content was determined by the method of Sagisaka (1976). Frond tissues (0.5g) were homogenized in 5% trichloroacetic acid and centrifuged at 17 000 *g* for 10 min at 0-4 °C and the homogenate was used for analysis. The level of H_2O_2 was expressed as mmol of H_2O_2 decomposed $min^{-1}g^{-1}$ fw at 25±2 °C.

Activity of antioxidant enzyme: Fronds of *Azolla* (0. 5g) were homogenized in ice cold 0.1 M, Tris – HCl buffer (pH 7.8) containing 0.1mM EDTA and made to a final volume of 10 ml. The homogenate was centrifuged at 12 000 *g* at 0-4 °C for 20 min and the supernatant was used as enzyme extract.

Activity of catalase (CAT; EC 1.11.1.6) was estimated as per the method of Chance and Maehly (1955). The 5.0 ml reaction mixture consisted of 3.0 ml phosphate buffer (pH 6.8), 1 ml (30 mM) H_2O_2 and 1 ml enzyme extract. The solution was stopped by adding 10 ml 2% H_2SO_4 after 1 min incubation at 20°C. The acidified reaction mixture was titrated against 0.01n$KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. The CAT activity was expressed as mmole of H_2O_2 min^{-1} g^{-1} fw at 25±2 °C. Peroxidase (POX; EC 1.11.1.7) activity was measured according to Chance and Maehly (1955). The 3.0 ml reaction mixture consisted of 0.1M phosphate buffer (pH 6.8), guaiacol (30 mM), H_2O_2 (30 mM) and 0.3 ml enzyme extract. The rate of absorbance at 420 nm was measured using UV–visible spectrophotometer. The peroxidase activity was expressed as mmole guaiacol oxidized $min^{-1}g^{-1}$fw at 25±2 °C.

The superoxide dismutase (SOD) activity was assayed by the method of Giannopolitis and Reis (1977). The reaction mixture contained 79.2 mM Tris–HCl buffer (pH 6.8), containing 0.12mM EDTA and 10.8mM, tetraethylenediamine, bovine serum albumin ($3.3x10^{-3}$%), 6mM nitroblue tetrazolium, 600 mM riboflavin in 5mM KOH and 0.2 ml enzyme extract. Reaction was initiated by placing the glass test tubes in between two fluorescent tubes (20W). The increase in absorbance due to formazan formation was read at 560 nm. Increase in absorbance in the absence of enzyme was taken 100% and 50% initially, an equivalent to 1 unit of SOD activity. Estimation of glutathione reductase (GR) was done as described by Smith *et al.* (1988). The reaction mixture consisted of 0.2M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.01M potassium phosphate buffer (pH 7.5), 2 mM NADPH, 1 ml enzyme extract and

distilled to make up a volume of 2.9 ml . Reaction was initiated by adding 2 mM oxidised glutathione. The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min. The activity was expressed as absorbance change (ΔA_{412}) per gram fw.

Determination of ascorbate and glutathione contents: Ascorbate (AsA) was determined according to the method of Oser (1977). The reaction mixture contained 2 ml 2% Na– molybdate, 2 ml 0.15N H_2SO_4, 1 ml 1.5 mM Na_2HPO_4 and 1 ml tissue extract, centrifuged at 14 000 *g* for 10min. and absorbance was measured at 660 nm. Glutathione (GSH) was assayed by the method of Griffith(1980). The tissue was homogenized in 5%(w/v) sulfosalicylic acid and homogenate was centrifuged at 12 000 g for 10 min. Total glutathione (GSH) was measured by adding 1 ml neutralized supernatant to a standard solution mixture consisting of 0.5 ml of 0.1M sodium phosphate buffer (pH 7.5) containing 1 ml of 1 mM ethylene diamine tetra acetic acid, 0.2 ml of 6 mM 5,5/- dithiobis (2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH and 0.1 cm^2 of 1 U ml^{-1} yeast GR type III (Sigma Chemical, USA.). The change in absorbance at 412 nm (ΔA_{412} g^{-1} FW), was followed at 25±2 °C until the absorbance reaches 0.5 units.

Bioaccumulation of chromium: The total chromium contents were analyzed as per the method of Humpries, (1956). The fronds of *Azolla* plants were dried at 80 °C for 48 h to a constant weight. The dried samples were then digested in 5 ml concentrated HNO_3 over a hot plate at 100 °C until the solution becomes clear. The final volume was adjusted to 20 ml by adding distilled water and the metal content was determined by atomic absorption spectroscopy (Perkin–Elmer 3110,Germany) as mg g^{-1}dw.

Statistical analysis: All the measurements were done in triplicates and data represent mean ± SE. Difference between control and treated plants was analyzed using Student's t-test, taking $P < 0.05$ as significant.

RESULTS

The accumulation of Cr [both Cr(III) and Cr(VI)] brought about an increase in concentrations of Cr in *Azolla* fronds (Fig.1). After 24h treatment, Cr(VI) showed 90% higher accumulation ($P < 0.05$) than Cr(III). The accumulation of Cr at higher concentration beyond the stipulated period of treatment (i.e. more than 6d) led to deterioration of plant. Both Cr(III) and Cr(VI) inhibited the growth of *Azolla* plants with significant decrease in dry weight (Table 3).The decrease in growth was maximum 17.9% at 10µM Cr(VI) whereas, in case of Cr(III) it was about 20.2% decrease at 10µM concentration after 2d of exposure.

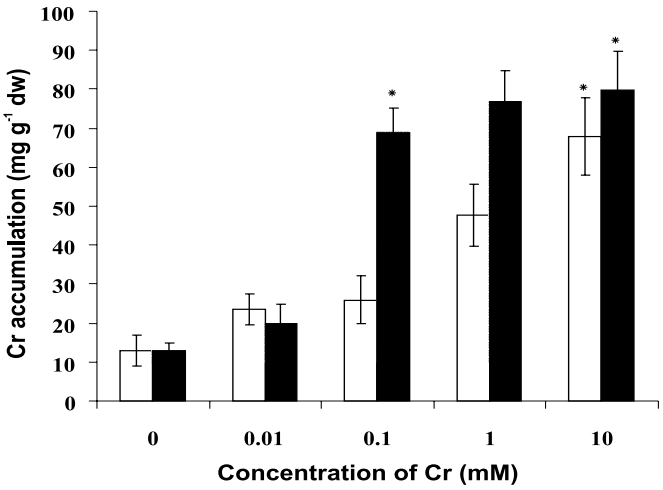

Fig. 1. Accumulation of Cr(III) [□] and Cr(VI) [■] in the fronds of *Azolla pinnata* under 24 h after chromium treatment. Data presented are means of three separate experiments ± SE and * indicates statistically significant differences in accumulation in comparison with non-treated *Azolla* fronds at $P < 0.05$

A significant increase in Malondialdehyde (MDA), total peroxide (H_2O_2), glutathione(GSH) and ascorbate (AsA) content was recorded (Table 1). The initial increase in MDA content [58.8% at 1mM Cr(III)] was seen on 6d with a maximum increase to 127.8% at 10µM Cr(VI) after 2d of exposure as compared to the untreated control. Similar increase [more than 4 fold at 10µM Cr(III)] of H_2O_2 was observed on the 6d of treatment.

Glutathione (GSH) and AsA contents also increased in fronds along with the increase in dose and duration of treatments. The GSH content increased by 459.6% at 10µM Cr(III). Higher concentrations of metal also increased the AsA content, as compared to the control plants.

Table 1. Changes in contents of MDA (mM malondialdehyde g^{-1} fw), H_2O_2 (mmol H_2O_2 decomposed min^{-1} g^{-1} fw), GSH (ΔA_{412} g^{-1} fw) and AsA (ΔA_{660} g^{-1} fw) in Cr (III) and Cr (VI) treated *Azolla* plants during 2, 4, 6 d exposure. Data are the mean value ± SD of three independent experiments with triplicates.
* indicates significant difference from the control plant at $P < 0.05$.

Parameters	Time (d)	Concentrations (µM)									
		Cr(III)					Cr(VI)				
		0	0.01	0.1	1	10	0	0.01	0.1	1	10
MDA	2	0.16±0.02	0.52±0.07	0.11±0.03	0.16±0.07	0.04±0.01	0.14±0.01	0.84±0.03	0.12±0.03	0.77±0.06	1.93±0.37
	4	0.02±0.07	0.40±0.07	0.03±0.05	0.12±0.03	0.09±0.11*	0.06±0.01	0.04±0.05	0.04±0.01	0.27±0.67*	0.03±0.01
	6	0.17±0.20	0.10±0.01	0.13±0.08	0.07±0.07	0.11±0.01*	0.44±0.29	0.70±0.26	0.20±0.04	0.20±0.01	0.21±0.01*
H_2O_2	2	1.58±0.04	16.41±0.67*	18.07±1.63*	45.17±0.13*	45.73±1.97*	1.43±0.07	5.90±0.08	12.33±0.37	12.97±0.43*	16.10±0.80*
	4	1.53±0.07	28.57±0.93*	35.17±4.67*	42.97±0.83*	69.83±1.77*	1.47±0.03	16.47±0.43*	21.67±0.67	31.39±0.31*	42.77±0.47*
	6	1.37±0.03	28.17±1.73*	46.37±0.83*	61.13±3.33*	66.33±11.37*	1.37±0.03	19.10±0.60*	36.77±0.27	39.77±2.03*	61.69±0.27*
GSH	2	3.33±0.13	25.84±6.73	19.11±6.73	31.94±15.54*	46.12±1.87*	2.83±0.37	39.77±9.16	53.13±4.97	58.13±0.03	63.23±1.42*
	4	3.44±0.05	38.67±4.18	33.47±6.73	57.34±9.69*	121.98±0.53*	5.03±1.63	1.60±0.60	5.77±2.50	17.90±4.20	22.10±4.20*
	6	2.91±0.217	66.49±8.84	75.38±8.84	115.16±3.97*	136.67±1.74*	3.23±0.63	36.17±1.73	73.60±3.40	75.50±4.90	116.83±2.37*
AsA	2	75.33±4.67	127.73±0.93*	196.27±0.78*	214.90±0.66*	257.57±0.83*	72.00±0.80	117.23±0.33*	147.10±0.05*	159.63±2.57*	206.01±2.97*
	4	54.64±0.70	111.17±0.47*	119.06±4.01*	202.38±0.67*	233.67±0.13*	54.57±0.73	86.43±0.33*	131.43±3.87*	132.03±4.27*	161.87±1.33*
	6	35.83±1.07	110.91±0.17*	111.69±0.07*	183.04±7.74*	200.47±3.36*	49.20±0.12	61.50±12.3*	121.38±1.33*	122.72±1.30*	147.95±1.95*

Table 2. Changes in activities of SOD (units g^{-1} fw), CAT (mmol H_2O_2 min^{-1} g^{-1} fw), POX (mmol guaiacol oxidised min^{-1} g^{-1} fw) and GR (ΔA_{412} g^{-1} fw) in Cr (III) and Cr (VI) treated *Azolla* plants during 2, 4, 6 d exposure. Data are the mean value ± SD of three independent experiments with triplicates.
* indicates significant difference from the control plant at $P < 0.05$.

Parameters	Time (d)	Concentrations (µM)									
		Cr(III)					Cr(VI)				
		0	0.01	0.1	1	10	0	0.01	0.1	1	10
SOD	2	0.27±0.03	0.37±0.03*	0.42±0.03*	0.51±0.03*	0.76±0.05*	0.28±0.03	0.37±0.02	0.44±0.03*	0.61±0.1*	0.79±0.02*
	4	0.23±0.07	0.45±0.03*	0.48±0.07*	0.63±0.17*	0.66±0.13*	0.23±0.07	0.39±0.03	0.47±0.02*	0.59±0.03*	0.73±0.03*
	6	0.39±0.08	0.61±0.07*	0.64±0.03*	0.61±0.14*	0.77±0.13*	0.36±0.07	0.56±0.17	0.80±0.02*	0.81±0.03*	0.73±0.17*
CAT	2	164.67±0.33	59.33±6.04*	67.20±4.20*	61.13±2.33*	15.10±0.04*	165.00±0.05	158.33±7.67*	69.77±7.13*	62.43±6.94*	49.00±10*
	4	213.00±1.01	74.47±8.84*	68.90±2.50*	52.30±3.20*	69.53±1.87*	216.33±2.33	84.40±6.50*	89.87±1.03*	68.30±3.10*	54.73±0.77*
	6	165.67±0.33	69.53±7.37*	66.43±0.17*	46.67±5.94*	46.33±6.27*	163.33±2.67	76.37±6.94*	69.67±1.73*	65.47±2.97*	64.77±9.27*
POX	2	5.35±0.28	12.97±0.84	25.31±0.10	34.65±0.19	51.90±3.25*	6.68±1.04	8.40±0.37	24.49±3.81	24.10±0.37	48.31±2.44*
	4	6.45±1.06	21.49±0.82	26.08±1.46	50.13±5.69	50.12±11.29*	8.35±0.83	9.81±0.27	36.96±12.52	33.07±2.37	59.83±2.23*
	6	7.63±0.65	76.50±13.5	126.35±1.67	130.50±0.13	158.25±3.40*	7.70±0.58	84.55±3.79	92.64±2.59	123.03±3.9	152.66±5.27*
GR	2	0.06±0.07	0.11±0.01	0.12±0.01	0.14±0.03*	2.10±0.43*	0.04±0.003	0.23±0.067	0.38±0.027	0.31±0.043*	0.40±0.01*
	4	0.04±0.08	0.42±0.08	0.39±0.03	0.90±0.02*	0.91±0.05*	0.05±0.013	0.25±0.167	0.12±0.007	0.50±0.08*	0.70±0.10*
	6	0.04±0.09	0.59±0.12	0.90±0.02	0.80±0.18*	1.39±0.03*	0.14±0.093	0.29±0.04	0.26±0.047	0.80±0.053*	0.91±0.03*

Table 3. Effect of Cr (III) and Cr (VI) on frond dry biomass production in *Azolla* plants during 2, 4, 6d exposure. Data are the mean value ± SD of three independent experiments with triplicates.
* indicates significant difference from the control plant at $P < 0.05$.

Parameters	Time (d)	Concentrations (µM)									
		Cr(III)					Cr(VI)				
		0	0.01	0.1	1	10	0	0.01	0.1	1	10
Dry Biomass (g)	2	0.20±0.02	0.28±0.03	0.24±0.05	0.21±0.03*	0.04±0.01	0.67±0.01	0.45±0.03*	0.12±0.03	0.11±0.06	0.12±0.37*
	4	0.19±0.01	0.11±0.01	0.10±0.05	0.09±0.01	0.09±0.01	0.76±0.01	0.54±0.05*	0.34±0.01*	0.27±0.07	0.13±0.01
	6	0.29±0.01	0.17±0.01	0.13±0.08	0.11±0.01	0.11±0.01*	0.84±0.29	0.70±0.24	0.20±0.04	0.20±0.01	0.18±0.01*

The activities of antioxidant enzymes (except CAT) SOD, POX and GR increased (Table 2). The SOD activity increased to 186.9% at 10μM Cr(III), but with continuous exposure it was 2 fold higher at 10μM after 4d of exposure. However, the CAT activity showed decreasing trends as compared to the untreated plants in both the chromium ions. The POX and GR activities increased by increasing the concentration of metal and duration of exposure. The maximum increase was after by 149.7% at 1μM Cr(III) in case of POX and 550.2% at 10μM Cr(VI) in GR after 6d of treatment in comparison to control.

DISCUSSION

It is seen that chromium changes the biochemical and metabolic responses in *Azolla pinnata* L. High bioaccumulation of Cr suggests the potential role of *A. pinnata* L. in phytoremediation. Similar findings have been reported for *A.caroliniana* and other species for their phytoaccumulation and phytoremediation properties (Shi *et al.* 2003, Bennicelli *et al.* 2004, Arora *et al.* 2006). The increasing Cr concentrations initiated an increase in the activity of antioxidative defense system in the plants. Hydrogen peroxide (H_2O_2) is produced as a result of enzymatic dismutation of $O_2^{\cdot-}$. Stress situation exacerbate H_2O_2 production in the apoplast, in chloroplasts and in other cellular compartments as investigated by Foyer *et al.*(1997). This kind of ROS is mobile and elevation of ROS content results in oxidative stress and the plant enzymatic antioxidant system is mobilized to remove the excess ROS. Lipid peroxidation measured in terms of TBARS, chiefly MDA content increased with the increasing concentration of Cr. This may be due to the generation of free radicals that may distort the membrane architecture causing an oxidative damage as reported in other higher plants (Shah *et al.* 2001, Kuo and Kao 2003, Sanita di Toppi *et al.*, 2004, Sinha *et al.* 2005).

The differences in SOD activity are dose-dependent (Siedlecka *et al.* 2000). The gradual increase in SOD activity with a simultaneous decline in CAT activity in *Azolla* indicated an accumulation of H_2O_2 which, in this case, suggested an imposition of oxidative stress (Dietz *et al.* 1999, Cakmak 2000, Breusegem *et al.* 2001). On the other hand, the POX and GR activities increased with each succeeding days of treatment as compared to control, thus suggesting their important role in preventing oxidative stress (Rennenberg 1982, Alscher 1989, Weckx and Clijster 1997). In addition, a gradual increase in GSH and AsA suggests their role in detoxification of ROS. Similar findings have been reported in other plants like *Brassica* by Prasad *et al.* (1999). With the increase in metal concentrations, physiological, biochemical and molecular changes are likely as reported in other aquatic plants (Bassi *et al.*, 1990, Panda and Patra 1997, Shi *et al.* 2003), and this is also substantiated by the induction of oxidative stress in the fronds of *Azolla*.

In conclusion, Cr can change the biochemical and metabolic processes in cells and the plant responses to higher concentrations of both Cr(III) and Cr(VI) – as for instance, A. pinnata L. was found to be more sensitive to Cr(VI) as compared to Cr(III) with a potential for phytoremediation in metal-polluted aquatic environment. The growth of *Azolla* plants under chromium can induce a concentration dependent oxidative stress situation in fronds. The generation and induction of oxidative stress could be a characteristic of the mechanism of Cr toxicity in plants. However, more information is needed at the sub-cellular and molecular levels in order to get deeper insights into the mechanism of Cr toxicity.

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